

## Exhibit B

- Lannoo, M. J., Brochu, G., Maler, L. & Hawkes, R. Zebrafish IL-1 immunoreactivity in the rat and in the weakly electric teleost *Eigmannia* (gnathostomes) reveals three modes of Putkin's cell
- McCourt, A. S. The control of neuronal identity in the developing cerebral cortex. *Cur. Opin. Neurobiol.* 2, 23–27 (1992).
- Goffinet, A. M. An early development defect in the cerebral cortex of the *reeler* mouse. A morphological study leading to a hypothesis concerning the action of the mutant gene. *Anat. Embryol.* 157, 205–216 (1976).
- Caviness, V. S. Jr Neurocritical histogenesis in normal and *reeler* mice: a developmental study based upon [3H]thymidine autoradiography. *Brain Res.* 256, 215–233 (1982).
- Hoffarth, R. M., Johnston, J. C., Krushel, L. A. & Van der Kooy, D. The *reeler* mutation *reeler* causes increased adhesion within a subpopulation of early postmitotic cortical neurons. *J. Neurosci.* 7, 4538–4552 (1987).
- Soriano, P. et al. Local control of granule cell generation by cerebellar Putkin's cells. *Mol. Cell. Neurosci.* 6, 250–251 (1995).
- Miyata, T. et al. Distribution of a *reeler* gene-related antigen in the developing cerebellum: an immunohistochemical study with an allelic antibody CR-50 on normal and *reeler* mice. *J. Comp. Neurol.* 372, 215–228 (1996).
- Soriano, P., Montgomery, C., Ceske, R. & Bradley, A. Targeted disruption of the c-scr proto-oncogene leads to osteopetrosis in mice. *Cell* 64, 693–702 (1991).
- Schwarzenberg, P. L. et al. Mice homozygous for a targeted mutation show poor viability and depletion of selected B and T cell populations. *Cell* 65, 1165–1175 (1991).
- Yan, J. C., Vlach, C. E., Jackson, P. K., Brune, R. T. & Mulligan, R. C. Neural lethality and lymphopenia in mice with a homozygous disruption of the c-scr proto-oncogene. *Cell* 65, 1163–1163 (1991).
- Sweet, H. O., Bruns, R. T., Johnson, K. R., Cook, S. A. & Davison, M. T. Scrambler, a new neurological mutation of the mouse with abnormalities of neural migration. *Mamm. Genome* 7, 798–802 (1996).
- Yoshimura, H. et al. A novel neurological mutation of mouse, *poteri* which has a *reeler*-like phenotype but expresses *reelin*. *Neurosci. Res.* (in press).
- Ohtsuka, T. et al. Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. *Proc. Natl. Acad. Sci. USA* 93, 11173–11178 (1996).
- Chen, T. et al. Mice lacking Cdk5, a neuronal specific activator of Cdk5, display cortical laminination defects, seizures, and adult lethality. *Neuron* 18, 29–42 (1997).
- Sheldón, M. et al. *Scrambler* and *poteri* disrupt the disabled gene and produce a *reeler*-like phenotype in mice. *Nature* 389, 730–733 (1997).
- Gerler, B. B., Hill, K. C., Clark, M. J. & Hoffmann, F. M. Dosage-sensitive modifiers of *Drosophila* akt tyrosine kinase function: *prospic*, a regulator of axonal outgrowth, and *disabled*, a novel tyrosine kinase substrate. *Genes Dev.* 7, 431–433 (1993).
- Gerler, B. B., Hill, K. C., Clark, M. J. & Hoffmann, F. M. *Drosophila* akt tyrosine kinase in *disabled*. *Nature* 369, 453–453 (1994).
- Elkins, T., Zinn, K., McAllister, L., Hoffmann, F. M. & Goodman, C. S. Genetic analysis of a *Drosophila* neural cell adhesion molecule interaction: *Psilolin* I and *Abedus* tyrosine kinase mutations. *Cell* 65, 565–575 (1991).
- Del Rio, J. A. et al. A role for Cajal–Retzius cells and reelin in the development of hippocampal connections. *Nature* 385, 70–74 (1997).

**Acknowledgements.** We thank R. Gerler and Y. Gotob for advice; T. Knight, P. Goodwin, C. Auger, E. González and L. O'Neal for technical assistance; K. Mikoshiba, T. Miyata and K. Nakajima for the CR-50 antibody and T. Curran and M. Sheldon for discussions. This work was supported by the NIH (J.A.C. and P.S.) and MRC Canada (R.H.).

Correspondence and requests for materials should be addressed to B.W.H. (e-mail: bhowell@fhcrc.org).

## ACD4<sup>+</sup> T-cell subset inhibits antigen-specific T-cell responses and prevents colitis

Hervé Groux, Anne O'Garra, Mike Bigler,  
Mathieu Rouleau, Svetlana Antonenko, Jan E. de Vries\* &  
Maria Grazia Roncarolo\*

DNAX Research Institute of Molecular and Cellular Biology, Inc.,  
Human Immunology Department, 901 California Avenue, Palo Alto,  
California 94304–1104, USA

Induction and maintenance of peripheral tolerance are important mechanisms to maintain the balance of the immune system. In addition to the deletion of T cells and their failure to respond in certain circumstances, active suppression mediated by T cells or T-cell factors has been proposed as a mechanism for maintaining peripheral tolerance<sup>1</sup>. However, the inability to isolate and clone regulatory T cells involved in antigen-specific inhibition of immune responses has made it difficult to understand the

mechanisms underlying such active suppression. Here we show that chronic activation of both human and murine CD4<sup>+</sup> T cells in the presence of interleukin (IL)-10 gives rise to CD4<sup>+</sup> T-cell clones with low proliferative capacity, producing high levels of IL-10, low levels of IL-2 and no IL-4. These antigen-specific T-cell clones suppress the proliferation of CD4<sup>+</sup> T cells in response to antigen, and prevent colitis induced in SCID mice by pathogenic CD4<sup>+</sup> CD45RB<sup>high</sup> splenic T cells. Thus IL-10 drives the generation of a CD4<sup>+</sup> T-cell subset, designated T regulatory cells 1 (Tr1), which suppresses antigen-specific immune responses and actively downregulates a pathological immune response *in vivo*.

Immune tolerance towards self antigens is dependent on the ability of the immune system to discriminate between self and non-self<sup>2</sup>. This occurs mainly through clonal deletion in the thymus of self-reactive T lymphocytes at an early stage of development. However, the immune system is also exposed to extrathymic self antigens and to repetitive stimulation by non-pathogenic antigens through inhalation and ingestion of foreign substances. To avoid chronic cell activation and inflammation, the immune system must develop unresponsiveness to such stimuli. Several mechanisms of peripheral tolerance have been proposed, including T-cell anergy<sup>3</sup>, T-cell deletion<sup>4</sup>, and active immune suppression<sup>5</sup>. Understanding these mechanisms of tolerance induction is clinically relevant for the treatment of autoimmune diseases and in transplantation, where the graft must ultimately be recognized as self.

The induction of anergy<sup>6</sup> and cell deletion by apoptosis<sup>2</sup> have been documented both *in vitro* and *in vivo*, but the analysis of active immune suppression mediated by T cells has been hampered by the inability to generate and clone these cells *in vitro*. The importance of cytokines in the development of specialized Th cells is now clear. Th1 cells are induced by activation in the presence of IL-12, whereas IL-4 drives the differentiation of Th2 cells<sup>6</sup>. Here we show that repetitive stimulation of CD4<sup>+</sup> T cells in the presence of IL-10 induces the differentiation of a unique subset of T cells with immunoregulatory properties.

ovalbumin (OVA)-specific naive CD4<sup>+</sup> T cells obtained from the  $\alpha\beta$  T-cell antigen receptor (TCR) DO11-10 transgenic mice<sup>7</sup> repeatedly stimulated with splenic antigen-presenting cells (APCs) and OVA peptide in the presence of IL-10, or IL-4 and IL-10, displayed a cytokine profile distinct from that of the classical Th0, Th1 or Th2 phenotype<sup>8</sup>. These T cells produce high levels of IL-10 and IL-5 and low levels of IL-2 and IL-4 (Fig. 1). Another characteristic of these T-cell populations is that they proliferated poorly in response to antigenic stimulation (Fig. 2a). In contrast, CD4<sup>+</sup> T cells isolated from OVA-specific TCR transgenic mice repeatedly stimulated with OVA peptide in the presence of IL-4 alone displayed the typical Th2-type profile, secreting IL-4, IL-5 and IL-10 (Fig. 1)<sup>9</sup>.

Analysis of T-cell clones isolated from the mouse CD4<sup>+</sup> T-cell populations that were repeatedly stimulated with OVA peptide and splenic APCs in the presence of IL-10 showed that half of the CD4<sup>+</sup> T-cell clones obtained displayed this cytokine profile. They produced high levels of IL-10 and undetectable levels of IL-2 and IL-4, whereas the levels of production of interferon (IFN)- $\gamma$  and transforming growth factor (TGF)- $\beta$  were comparable with those of Th0 and Th1 clones, respectively (Table 1). These T-cell clones also proliferated poorly in response to antigen-specific stimulation (Fig. 2a), which may explain the previous failure to isolate these cells. Only Th1, Th2 and Th0 clones were isolated from T-cell populations cultured in the absence of IL-10 (Table 1). Thus chronic activation of mouse CD4<sup>+</sup> T cells in the presence of IL-10 results in the generation of a T-cell subset with a unique cytokine profile and low proliferative capacity. These cells are designated Tr1 cells on the basis of their function.

Tr1 cells can also be generated from human peripheral blood. Both alloantigen-specific CD4<sup>+</sup> T-cell clones (JDV24) and non-alloantigen-specific T-cell clones (JDV15, JDV308 and JDV94)

\* Present address: Novartis Research Institute, Brunner Strasse 59, A-1235 Wien, Austria (J.E.D.V.); University of Torino, Department of Pediatrics, Regina Margherita Children's Hospital, Piazza Polonia 94, 10126 Torino, Italy (M.G.R.).

displaying the Tr1 cell cytokine profile were derived from CD4<sup>+</sup> T-cell populations that had initially been stimulated with allogeneic monocytes in the presence of IL-10 (Table 2). In contrast, only Th1-type clones were obtained when the CD4<sup>+</sup> T cells were stimulated by allogeneic monocytes in the absence of IL-10. The most striking characteristic of the human Tr1 cell clones was their unusually high level of IL-10 production (Table 2). They also produced very low levels of IL-2, and failed to secrete detectable levels of IL-4, whereas their levels of IL-5, IFN- $\gamma$  and TGF- $\beta$  production were similar to those of human Th0 clones. Taken together, these results demonstrate that antigen-specific Tr1 cells can be generated *in vitro* from naïve populations of both human and mouse CD4<sup>+</sup> T cells.

Human Tr1 clones stimulated with either immobilized anti-CD3 and anti-CD28 monoclonal antibodies or allogeneic monocytes (for

JDV24) showed low proliferative responses that were sustained by secretion of IL-2, as the addition of an anti-IL-2 monoclonal antibody completely blocked the growth of the Tr1 cells (Fig. 2b). Kinetic studies showed that IL-10 is produced rapidly after activation of the human Tr1 clones. IL-10 is detectable in the supernatants of Tr1 clones 8 h after activation (data not shown), which indicates that IL-10 production by Tr1 clones generally occurs before or at the same time as IL-2 production. In contrast, production of IL-10 by Th0, Th1 and Th2 clones established from the same donor in the same experiment was not detectable until 20 h after activation, which is compatible with previous findings<sup>15</sup>.

Early endogenous IL-10 production accounts in part for the low proliferative capacity of the human and mouse Tr1 clones in response to TCR/CD3 stimulation (Fig. 2), as proliferation of the

Table 1 Cytokine production by murine OVA-specific CD4<sup>+</sup> T-cell clones

T-cell clones	Initial incubation with IL-10	IL-2 (pg ml <sup>-1</sup> )	IL-4 (pg ml <sup>-1</sup> )	IL-10 (pg ml <sup>-1</sup> )	IFN- $\gamma$ (ng ml <sup>-1</sup> )	TGF- $\beta$ (pg ml <sup>-1</sup> )	Clone type
A-10-1	yes	<40	<50	1,230 ± 310	106 ± 27	—	Tr1
A-10-9	yes	<40	<50	1,020 ± 120	87 ± 17	435 ± 9	Tr1
A-10-11	yes	<40	<50	1,850 ± 410	217 ± 68	747 ± 9	Tr1
A-10-14	yes	<40	<50	1,820 ± 120	198 ± 67	—	Th0
A-10-2	yes	102 ± 26	250 ± 45	850 ± 130	488 ± 124	—	Th0
A-10-7	yes	97 ± 27	897 ± 102	410 ± 120	267 ± 247	—	Th0
A-10-15	yes	52 ± 19	89 ± 17	205 ± 80	420 ± 11	—	Th0
A-10-19	yes	69 ± 27	890 ± 48	310 ± 120	107 ± 57	—	Th0
A-3	no	<40	1,247 ± 157	510 ± 110	<75	—	Th2
A-5	no	<40	879 ± 87	310 ± 60	<75	—	Th2
A-7	no	129 ± 48	<50	—	375 ± 97	715 ± 2	Th1
A-13	no	89 ± 28	598 ± 59	215 ± 30	143 ± 13	—	Th0
A-14	no	203 ± 49	<50	<75	248 ± 49	—	Th1

Cytokine production by murine OVA-specific CD4<sup>+</sup> T-cell clones derived from cells of DO11.10 TCR<sup>+</sup> transgenic mice repetitively stimulated in the presence or absence of IL-10. Naive (MEL14) (bright) CD4<sup>+</sup> T cells from DO11.10 transgenic mice were stimulated with OVA peptide (0.6  $\mu$ M) in the presence (A-10) or absence (A-11) of IL-10 (100 U ml<sup>-1</sup>). After repetitive stimulations under the same conditions, the CD4<sup>+</sup> T cells from these two populations were cloned by limiting dilution. After expansion, the T-cell clones were stimulated by OVA peptide (1  $\mu$ M) and irradiated total splenic APCs. Cytokines were analysed by ELISA in culture supernatants collected after 48 or 72 h of culture for TGF- $\beta$ . Results represent pooled data from 4 representative experiments.

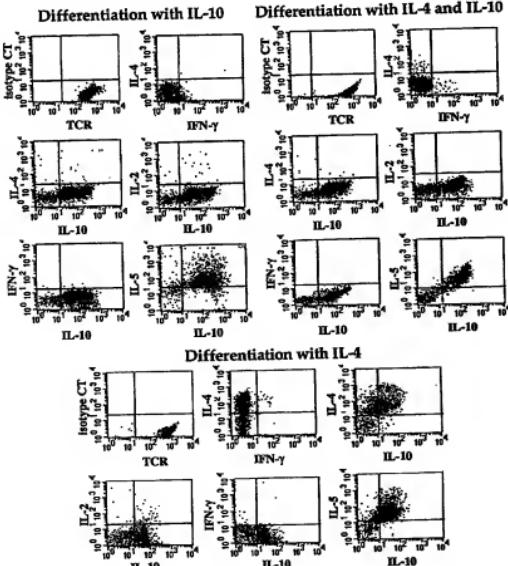
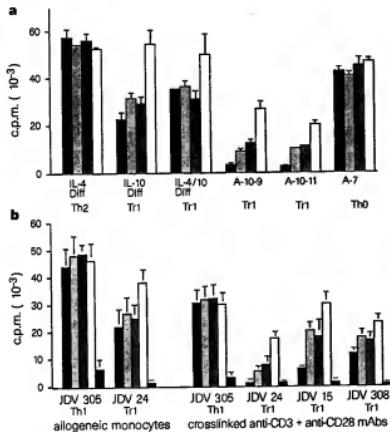


Figure 1 Generation of murine Tr1 cells in the presence of IL-10. Naive (MEL14) (bright) CD4<sup>+</sup> T cells from BALB/c mice transgenic for the DO11.10 α $\beta$  TCR<sup>+</sup> were cultured with OVA peptide (0.6  $\mu$ M) and irradiated splenic APCs in the presence or absence of IL-4 (200 U ml<sup>-1</sup>), IL-10 (100 U ml<sup>-1</sup>), or both. The stimulation, under the same conditions, was repeated weekly for three consecutive weeks<sup>2</sup>. Cells were collected, washed, and restimulated with immobilized anti-CD3 (10  $\mu$ g ml<sup>-1</sup>) and anti-CD28 mAb (10  $\mu$ g ml<sup>-1</sup>) for 4 h at 37°C. Brefeldin A (10  $\mu$ g ml<sup>-1</sup>) was added for the last 2 h of culture<sup>9</sup>. The cells were fixed and stained for detection of intracellular cytokines using FITC or PE-conjugated monoclonal antibodies specific for IFN- $\gamma$ , IL-2, IL-4, IL-5 and IL-10, or an isotype-matched control mAb (isotype CT), as indicated. Cells were also stained with the anti-TCR clone-specific mAb KJ-1-26 (TCR) to show that all cells were positive for the transgene after 3 weeks of culture. One of three experiments is shown.

Tr1 clones was augmented by the addition of a neutralizing anti-IL-10 monoclonal antibody, which is consistent with previous observations indicating that IL-10 prevents or inhibits T-cell proliferation<sup>11</sup>. In contrast, anti-IL-10 monoclonal antibody had no effect on the proliferative responses of control Th1, Th2 or Th0 clones (Fig. 2). Proliferation of both human and mouse Tr1 clones was also partly augmented by a neutralizing anti-TGF- $\beta$  monoclonal antibody (Fig. 2), whereas the proliferation of the control Th clones was unaffected (Fig. 2). The combination of anti-IL-10 and anti-TGF- $\beta$  antibodies had additive effects and almost completely restored the proliferative responses of Tr1 clones (Fig. 2).

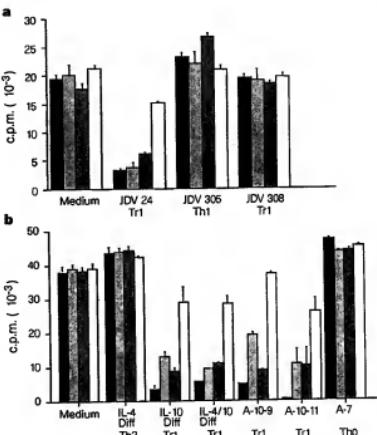
The observation that Tr1 cells secrete high levels of the immunosuppressive cytokine IL-10, and low levels of the T-cell growth-promoting cytokines IL-2 and IL-4, suggested that antigen-specific activation of Tr1 cells may result in inhibition of antigen-specific proliferation of other T cells. Indeed, co-culture experiments using a transwell system confirmed this notion for both human and mouse T cells. Resting human CD4 $^+$  T cells were stimulated with irradiated allogeneic monocytes in the bottom compartment, whereas alloantigen-specific Tr1 clone JDV24 or non-antigen-specific Tr1 clone



**Figure 2** Proliferative responses of Tr1 cells. **a**, Naive (MEL14 bright) CD4 $^+$  T cells from BALB/c mice transgenic for the DO110 $\alpha\beta$  TCR $^+$  were differentiated (Dif) *in vitro* as described in Fig. 1 and restimulated with OVA peptide (0.6  $\mu$ M) and irradiated splenic APCs for 3 days. One OVA-specific Th0 clone (A-7) and two OVA-specific Tr1 type clones (A-10-9 and A-10-11) were stimulated using the same conditions. **b**, The human Th1 (JDV 305) and Tr1 (JDV24) alloantigen-specific CD4 $^+$  T-cell clones or the non-alloantigen-specific Tr1 type clones (JDV15 and JDV 308) were stimulated with either allogeneic irradiated monocytes for 5 days or with immobilized anti-CD3 (10  $\mu$ g ml $^{-1}$ ) and anti-CD28 mAb (1  $\mu$ g ml $^{-1}$ ) for 3 days. The proliferative responses were measured by [ $^3$ H]-TdR incorporation during the last 12 h of culture. Cultures of human or mouse T cells were carried out in the presence of an isotype-matched control mAb (GL113, rat IgG1 at 20  $\mu$ g ml $^{-1}$  + mouse IgG1 from Caltag (Burling, CA) at 20  $\mu$ g ml $^{-1}$ ; black bars); a neutralizing anti-IL-10 mAb (12G8 at 5  $\mu$ g ml $^{-1}$  for human T cells or JE55-2A5 at 10  $\mu$ g ml $^{-1}$  for mouse T cells; white hatched bars); a neutralizing anti-TGF- $\beta$  (from Genzyme (Cambridge, MA) at 10  $\mu$ g ml $^{-1}$ ; black hatched bars); a combination of the two antibodies (white bars); or a neutralizing anti-IL-2 mAb (BG-5, 10  $\mu$ g ml $^{-1}$  for human cells; grey bars). A representative Tr1 (JDV 305) and a representative Tr1 (JDV24) are shown for antigen-specific controls. One of four experiments is shown.

JDV308 were stimulated with the same allogeneic monocytes in the top compartment. Alloantigen-specific stimulation of the Tr1 clone JDV24 strongly reduced the alloantigen-specific proliferative responses of autologous human CD4 $^+$  T cells, whereas the non-alloantigen-specific Tr1 clone JDV308 was ineffective (Fig. 3a). A slight increase in CD4 $^+$  T-cell proliferation was noticed following co-culture with the alloantigen-specific T-cell clone JDV305, which belongs to the Th1 subset (Fig. 3a).

Similar data were obtained with mouse Tr1 clones (Fig. 3b). The proliferation of naive CD4 $^+$  T cells in response to OVA peptide and splenic APCs was dramatically reduced following co-culture in the transwell system with OVA peptide-activated Tr1 clones or with activated Tr1-cell populations generated in the presence of IL-10. In contrast, OVA-specific Th0 clones or Th2-cell populations generated in

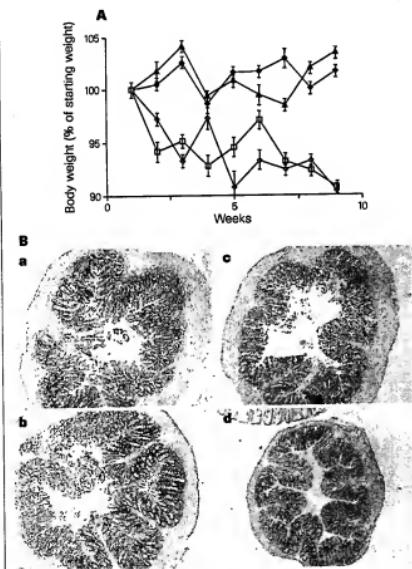


**Figure 3** Tr1 cells inhibit the antigen-specific proliferative responses of naive CD4 $^+$  T cells. **a**, Purified human CD4 $^+$  T cells from donor JDV were stimulated by allogeneic purified irradiated monocytes for 5 days in the bottom compartment of a transwell system. The resting T cells were co-cultured with various autologous T-cell clones contained in the upper transwell compartment; the Tr1 clone JDV24 and the Th1 clone JDV305 are alloantigen-specific, whereas the Tr1 clone JDV308 is non-alloantigen-specific. After 5 days the basket was removed and the proliferative response of the CD4 $^+$  T cells was analysed by [ $^3$ H]-TdR incorporation during the last 12 h of culture. **b**, Naive (MEL14 bright) CD4 $^+$  T cells from BALB/c mice transgenic for the DO110 $\alpha\beta$  TCR were stimulated in the bottom compartment of a transwell system with OVA peptide (0.6  $\mu$ M) and irradiated APCs for 3 days. These naive T cells were co-cultured with the indicated populations contained in the upper transwell basket. OVA-specific CD4 $^+$  T-cell populations differentiated (dift) *in vitro* by three consecutive restimulations in the presence of IL-4 (IL-4 Dif), IL-10 (IL-10 Dif) or IL-4 and IL-10 (IL-4/10 Dif), respectively, or OVA-specific CD4 $^+$  T-cell clones A-10-9, A-10-11 (Tr1 type clones) or A-7 (Th0 type clone). After 3 days the basket was removed and the proliferative response of the naive CD4 $^+$  T cells was analysed by [ $^3$ H]-TdR incorporation during the last 12 h of the culture. Cultures of human and murine cells were carried out in the presence of an isotype-matched control (GL113, rat IgG1 at 20  $\mu$ g ml $^{-1}$  + mouse IgG1 from Caltag (Burlingame, CA) at 20  $\mu$ g ml $^{-1}$ ; black bars); a blocking anti-IL-10 mAb (12G8 at 5  $\mu$ g ml $^{-1}$  for human cells or JE55-2A5 at 10  $\mu$ g ml $^{-1}$  for mouse cells; white hatched bars); a blocking anti-TGF- $\beta$  (from Genzyme (Cambridge, MA) at 10  $\mu$ g ml $^{-1}$ ; black hatched bars); or a combination of the two antibodies (white bars). Results represent one of three experiments performed.

Table 2 Cytokine production by human Tr1 cells

T-cell clones	Clone type	IL-2	IL-4	IL-5	IL-10	IFN- $\gamma$	TGF- $\beta$
Cells preincubated with IL-10							
JDV15	Tr1	<20	<40	5,210	11,620	1,370	220
JDV24	Tr1	<20	<40	12,540	14,370	420	230
JDV308	Tr1	70	<40	8,450	12,630	720	510
JDV84	Tr1	<20	<40	6,670	12,470	110	
JDV81	Th0	420	2,140	6,250	510	1,680	
JDV6	Th1	640	<40	90	720	6,480	
JDV10	Th1	420	<40	<40	640	4,290	
Cells preincubated without IL-10							
JDV305	Th1	140	<40	1,220	450	2,520	220
JDV5	Th1	850	<40	<40	1,220	8,450	
JDV40	Th1	480	<40	210	720	4,240	
JDV60	Th1	570	<40	330	440	3,580	
JDV101	Th1	440	<40	150	320	6,540	

To generate antigen-specific T-cell clones, CD4<sup>+</sup> T cells from donor JDV cells were stimulated with irradiated purified allogeneic monocytes in the presence or absence of IL-10 (100 U ml<sup>-1</sup>). After 10 days, CD4<sup>+</sup> T cells were cloned. The different T-cell clones were expanded and selected for their specificity against the same allogeneic monocytes. To measure cytokine production, T cells were activated with crosslinked anti-CD3 (10  $\mu$ g ml<sup>-1</sup>) and anti-CD28 (1  $\mu$ g ml<sup>-1</sup>) mAbs, supernatants were collected after 48 h of culture or 72 h for TGF- $\beta$ . Results are expressed in pg ml<sup>-1</sup> and represent pooled data from four independent experiments.



**Figure 4** Co-transfer of Tr1 clones prevents colitis. **A**, Groups of 5 acid mice were reconstituted with  $4 \times 10^6$  CD4<sup>+</sup>CD45RB<sup>hi</sup> splenic T cells from 6-week-old BALB/c mice either alone (white squares) or in the presence of either  $2 \times 10^6$  CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells (black triangles) or  $2 \times 10^6$  OVA-specific Tr1 clones (A-10-9) (white diamonds for animal not fed with ovalbumin, and black diamonds for animals fed with ovalbumin). Half of the groups were fed with ovalbumin diluted in the drinking water (100 ng ml<sup>-1</sup>). Feeding with ovalbumin did not change the pathogenic effect of CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells or the protective effect of CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells, and only animals fed with ovalbumin are shown for these two groups. Data represent mean  $\pm$  s.e.m. of 15 animals per group. Results are representative of two experiments. **B**, Severe colitis in mice restored with CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells (**a**) and with CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells and a Tr1 clone (A-10-9) but not fed with ovalbumin (**b**). Protection from colitis in mice reconstituted with either CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells plus CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells (**c**) or in mice reconstituted with CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells and a Tr1 clone fed with ovalbumin (**d**).

the presence of IL-4 had no suppressive effects, but rather enhanced OVA-induced proliferation of naive CD4<sup>+</sup> T cells.

Addition of neutralizing anti-IL-10 and anti-TGF- $\beta$  antibodies augmented the proliferative responses of naive human and mouse CD4<sup>+</sup> T cells in the presence of Tr1 cells (Fig. 3). These data demonstrate that IL-10 induces the *in vitro* differentiation of a new regulatory CD4<sup>+</sup> T-cell subset, which suppresses antigen-specific T-cell responses in both mice and man. These suppressive activities are predominantly mediated by IL-10 and TGF- $\beta$ . Moreover, similar to the data previously reported for T cells stimulated in the presence of exogenous IL-10 (ref. 12), naive T cells stimulated in the presence of Tr1 clones fail to proliferate and to secrete IL-2, IL-4, IL-5 and IFN- $\gamma$  after re-stimulation (not shown).

We sought to establish whether these Tr1 clones were functional *in vivo* and could regulate a pathogenic T-cell response. IL-10 is important for the maintenance of T-cell infiltration in the gut, as IL-10-deficient mice develop inflammatory bowel disease, which is thought to be mediated by normal enteric antigens<sup>13</sup>. In addition, administration of IL-10 to young IL-10-deficient mice prevents inflammatory bowel disease<sup>14</sup>. IL-10 also significantly inhibits the development of inflammatory bowel disease (IBD) induced by the transfer of CD4<sup>+</sup>CD45RB<sup>hi</sup> D4<sup>+</sup> T cells into SCID mice<sup>15</sup>. Here we show, in this latter model of IBD induction, that transfer in SCID mice of as few as  $2 \times 10^5$  OVA-specific Tr1 cells (A-10-9 (Fig. 4) or A-10-11 (data not shown)) prevents IBD induced by pathogenic CD4<sup>+</sup>CD45RB<sup>hi</sup> splenic T cells. Not surprisingly, the Tr1 cells are effective only upon stimulation *in vivo* by feeding the mice with OVA (Fig. 4). No difference in the pathogenicity of the CD4<sup>+</sup>CD45RB<sup>hi</sup> splenic T cells or in the protective effects of the CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells<sup>16</sup> was observed after feeding the mice with OVA in the absence of Tr1 cells (Fig. 4). These results demonstrate that Tr1 clones can prevent a T-cell-mediated disease *in vivo*. Furthermore, they indicate that the Tr1 cells must be activated *in vivo* to be effective. Finally, because the function of Tr1 cells is mediated by soluble factors, the present findings suggest that they can suppress active immune responses to unknown antigens in the microenvironment by an antigen-driven bystander suppression mechanism.

Transfer of CD4<sup>+</sup> T-cell clones isolated from the gut of SJL mice after oral tolerance induction with myelin basic protein (MBP)<sup>17</sup> or from the pancreas of NOD mice<sup>18</sup> has been shown to suppress experimental autoimmune myelitis and diabetes, respectively. These inhibitory activities were relieved by anti-TGF- $\beta$ , suggesting that these clones mediated their suppression through TGF- $\beta$ <sup>17,18</sup>. Although these T-cell clones appear to have similar functions to Tr1 cells, their cytokine production profiles are different. The T-cell clones isolated from SJL and NOD mice produce low levels of IL-10 and high levels of biologically active TGF- $\beta$ , whereas the T-cell clones isolated from MBP transgenic mice fed with MBP (called Th3

cells) exclusively produced active TGF- $\beta$  and no IL-10, IL-4, IL-2 or TNF- $\alpha$ <sup>19</sup>. In contrast, Tr1 cells consistently produce low levels of active TGF- $\beta$  and high levels of IL-10. Another difference is that both cytokines contribute to the suppressive activity of Tr1 cells. Taken together, these data suggest that Th3 and Tr1 cells are distinct types of CD4 $^{+}$  regulatory T cells.

The isolation of Tr1 cells from the peripheral blood of SCID patients, in which high levels of IL-10 *in vivo* are associated with tolerance after allogeneic stem-cell transplantation<sup>18</sup>, and from anergic T cells stimulated by major histocompatibility complex (MHC) class II-positive melanoma cells<sup>20</sup>, supports the hypothesis that Tr1 cells are generated *in vivo* by chronic exposure of naïve T cells to alloantigens in the presence of IL-10. Furthermore, based on the *in vitro* immunosuppressive activities of Tr1 cells, it is possible that these Tr1 cells specific for the host antigens have similar properties *in vivo*, and therefore may be responsible for transplantation tolerance in man. The observation that IL-10 production is associated with tolerance in murine models of organ and stem-cell transplantation is consistent with this notion<sup>21,22</sup>.

However, administration of IL-10 failed to induce T-cell tolerance in other experimental models of transplantation<sup>23–26</sup> and autoimmune disease, such as experimental autoimmune encephalomyelitis<sup>27</sup> or autoimmune haemolytic anaemia<sup>28</sup>, which could be explained by our observations suggesting that the potential induction of tolerance through differentiation of Tr1 cells is a lengthy process *in vivo*, requiring chronic antigen-specific stimulation in the presence of IL-10. Thus systemic administration of IL-10 after the onset of the disease may not impede the effector phase of the immune response, and may therefore not result in tolerance.

Our findings indicate that stimulation of CD4 $^{+}$  T cells in the presence of IL-10 generates a subset of CD4 $^{+}$  T cells that inhibits antigen-specific immune responses through the secretion of IL-10 and TGF- $\beta$ . The cytokine profile appears to be stable, as Tr1 clones cultured for more than 12 months produced the same cytokine profile after activation. All regulatory cells described previously have been isolated after *in vivo* priming with antigens. The ability to generate Tr1 cells after exposure to antigen *in vitro* may be an advantage for further functional analysis and potential clinical applications, particularly because we have demonstrated their regulatory capacity *in vivo*. Moreover, because Tr1 cells suppress immune responses directed against other antigens in the microenvironment, they could potentially be used to regulate T-cell-mediated disease, even when the pathogenic antigen is unknown. □

#### Methods

**Cloning of Tr1 cells.** Human Tr1 cells were generated from peripheral blood CD4 $^{+}$  T cells stimulated with allogeneic monocytes in the presence of exogenous IL-10. After 10 days, the CD4 $^{+}$  T cells were cloned by flow cytometry. Cells were labelled with an anti-CD4-FITC monoclonal antibody (Becton Dickinson, Mountain View, CA) and the viable cells were sorted at one cell per well in wells pre-coated with anti-CD3 monoclonal antibody (SPV-T3; ref. 12) 100  $\mu$ g  $ml^{-1}$  in 100  $\mu$ l Tris, 0.1 M, pH 9.4. After sorting, a mixture of irradiated feeder cells (10 $^{5}$  per ml and PBMC, 10 $^{6}$  per ml) and 10 U  $ml^{-1}$  of IL-2 in 100  $\mu$ l were added. Clones were expanded with IL-2 (10 U  $ml^{-1}$ ) and by repetitive stimulation with high doses of crosslinked anti-CD3 monoclonal antibody (100  $\mu$ g  $ml^{-1}$ ). The cloning of the mouse Tr1 cells was performed by limiting dilution at 0.3 cells per well in wells coated with 50  $\mu$ g  $ml^{-1}$  of anti-CD3 monoclonal antibody (2C11, PharMingen, San Diego) in PBS and containing irradiated total splenocytes (10 $^{7}$  per ml) and 20 U  $ml^{-1}$  IL-2. Clones were expanded by culture with IL-2 (20 U  $ml^{-1}$ ) and IL-4 (20 U  $ml^{-1}$ ), OVA peptide (2  $\mu$ M) and irradiated splenic APCs (10 $^{7}$  per ml).

**Flow cytometry analysis of intracellular cytokines.** Analysis of intracellular cytokines by flow cytometry was performed as described<sup>9</sup>. Cells (10 $^6$  per ml) were activated with immobilized anti-CD3 and anti-CD28 monoclonal antibodies (PharMingen, San Diego) for 4 h. Brefeldin A (Sigma, St Louis) was added at 10  $\mu$ g  $ml^{-1}$ , and 2 h later cells were collected, washed and fixed with

formaldehyde (2%). For intracellular staining, cells were incubated with the following monoclonal antibodies (PharMingen, San Diego): anti-IL-4-FITC or PE (11B1, 5  $\mu$ g  $ml^{-1}$ ), anti-IFN- $\gamma$ -PE (XMG1.2, 5  $\mu$ g  $ml^{-1}$ ), anti-IL-5-PE (TRFK5-2, 5  $\mu$ g  $ml^{-1}$ ), anti-IL-10-FITC (JES-16E3, 5  $\mu$ g  $ml^{-1}$ ), anti-IL-2-PE (JES-5H4, 2.5  $\mu$ g  $ml^{-1}$ ) or the anti-clonotype KJ1-26-FITC (5  $\mu$ g  $ml^{-1}$ ). Samples were analysed on a FACSscan (Becton Dickinson, Mountain View, CA).

**Cytokine analysis.** Human CD4 $^{+}$  T-cell clones or populations at 1  $\times$  10 $^6$  per ml were stimulated by immobilized anti-CD3 (10  $\mu$ g  $ml^{-1}$ ) and anti-CD28 monoclonal antibodies (1  $\mu$ g  $ml^{-1}$ ) for 48 h. The production of IL-2, IL-4, IL-5, IL-10 and IFN- $\gamma$  was measured by immunoenzymatic assays, as described<sup>12</sup>. The amounts of IL-2, IL-4, IL-10 and IFN- $\gamma$  produced by murine CD4 $^{+}$  T-cell clones was measured by ELISA in supernatants collected 48 h after culture of the T cells (10 $^6$  per ml) stimulated with OVA-peptide (1  $\mu$ M) and irradiated total splenic APCs, as described<sup>9</sup>. For TGF- $\beta$  measurements, cells were cultured for 72 h in Yssel's medium<sup>29</sup> without serum and the amount of TGF- $\beta$  was measured by ELISA (R and D System, Minneapolis, MN) after an acid activation, according to the manufacturer's instructions.

**Proliferation assays.** All proliferation assays were carried out in Yssel's medium<sup>29</sup> supplemented with 10% FCS and 1% human serum for human cells or with 10% FCS for murine cells. Alloantigen-specific stimulation was performed by culturing human CD4 $^{+}$  T-cell clones (10 $^6$  per ml) with purified irradiated (4,000 rad) monocytes (10 $^6$  per ml) in 200  $\mu$ l round-bottomed 96-well plates (Linbro, ICN Biomedicals, Aurora, OH). T-cell proliferation was measured after 5 days incubation at 37 °C and 5% CO<sub>2</sub> and a 12-h pulse with [<sup>3</sup>H]-TdR. Activation of human CD4 $^{+}$  T cells with immobilized anti-CD3 and anti-CD28 mAb was performed as described<sup>12</sup>. In brief, antibodies were diluted at the indicated concentration in 0.1 M Tris buffer, pH 9.4, and plates incubated for a week at 4 °C. After washing the plates, CD4 $^{+}$  T cells were added at a concentration of 5  $\times$  10 $^4$  cells per well and proliferation was measured after 3 days by [<sup>3</sup>H]-TdR uptake. Similarly, murine CD4 $^{+}$  T-cell populations or T-cell clones were stimulated at a concentration of 5  $\times$  10 $^4$  cells per well with OVA peptide (0.5  $\mu$ M) and irradiated (4,000 rad) total splenocytes (5  $\times$  10 $^5$  cells per well) for 3 days.

**Inflammatory bowel disease.** The induction of IBD was performed as described<sup>30</sup>. In brief, C57BL/6J mice 8–12 weeks old (Simonsen Laboratories, Gilroy, CA) were injected intraperitoneally with 100  $\mu$ l of PBS containing 4  $\times$  10 $^5$  CD4 $^{+}$ CD45RB<sup>hi</sup> sorted splenic T cells with or without either 2  $\times$  10 $^5$  CD4 $^{+}$ CD45RB<sup>hi</sup> splenic T cells or 2  $\times$  10 $^5$  Tr1 clones. Half of the mice were fed with OVA diluted in the drinking water at 100 ng  $ml^{-1}$ . Weight gain or loss, as a percentage of original starting weight, was scored weekly for 9 weeks. For preparation of tissue for histopathologic analysis, approximately 10-mm segments of the distal large intestine were removed and fixed in a solution of 10% formalin. Fixed tissues were frozen and sections were prepared and stained with haematoxylin.

Received 16 June; accepted 8 August 1997.

1. Benacerraf, B. & Germain, R. N. A single major pathway of T-lymphocyte interactions in antigen-specific immune responses. *Scand. J. Immunol.* 15, 1–10 (1981).
2. Beuken, B. & von Boehmer, H. Peripheral selection of the T cell repertoire. *Science* 251, 1225–1228 (1991).
3. Nossal, G. J. Bone marrow pre-B cells and the clonal anergy theory of immunologic tolerance. *Int. Rev. Immunol.* 2, 321–338 (1987).
4. Jenkins, M. K., Chen, C. A., Jung, C. G., Mueller, D. L. & Schwartz, R. H. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J. Immunol.* 144, 16–22 (1990).
5. Schwartz, R. H. A cell culture model for T lymphocyte clonal anergy. *Science* 248, 1349–1356 (1990).
6. O'Garra, A. & Murphy, K. Role of cytokines in development of Th1 and Th2 cells. *Chim. Immunol.* 63, 1–13 (1994).
7. Murphy, K. M., Heinzenberger, A. B. & Loh, D. Y. Induction by antigen of thymic apoptosis of CD4 $^{+}$ CD45RB $^{hi}$  T-cell clones. *Science* 258, 1720–1722 (1990).
8. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedd, M. A. & Coffman, R. L. Two types of murine T-cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136, 2348–2357 (1986).
9. Murphy, K. E. et al. Requirement for Th1 and 2 populations is lost after long-term stimulation. *J. Exp. Med.* 183, 901–913 (1996).
10. Borsig, L. et al. High levels of interleukin 10 production *in vivo* are associated with tolerance in mice transplanted with HLA mismatched hematopoietic stem cells. *J. Exp. Med.* 179, 493–503 (1994).
11. de Weerdt Malefyt, R. et al. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via down-regulation of class II major histocompatibility complex expression. *J. Exp. Med.* 174, 915–924 (1991).
12. Groux, H., Bigler, M. B. & Roncarolo, M. G. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4 $^{+}$  T cells. *J. Exp. Med.* 184, 19–29 (1996).
13. Renick, D., Berg, D., Kahn, R. & Miller, W. In: *Interleukin-10* (ed. de Vries, J. E. & de Weerdt Malefyt, R.) 141–148 (R. G. Landes, Austin, TX, 1995).

14. Kuhn, R., Lohrer, J., Rennick, D., Rajewski, K. & Muller, W. Interleukin-10 deficient mice develop chronic enterocolitis. *Cell* 75, 263–274 (1993).

15. Powrie, F. et al. Induction of chronic spontaneous inflammatory bowel disease in scid mice requires CD14<sup>+</sup> CD14<sup>+</sup> cells. *Immunity* 7, 535–542 (1994).

16. Powrie, F., Lynch, M. W., Maestre, S., Castle, L. B. & Coffman, R. L. Phenotypically distinct subsets of CD4<sup>+</sup> T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int. Immunopharmacol.* 5, 1461–1471 (1993).

17. Chen, Y., Kuchroo, V. K., Inobe, J.-I., Hafler, D. A. & Weiner, H. L. Regulatory T cells induced by oral tolerance suppression of autoimmune encephalomyelitis. *Science* 265, 1237–1240 (1994).

18. Han, H. S., Jun, H. S., Utsugi, T. & Yoon, J. W. A new type of CD4<sup>+</sup> suppressor T cells completely prevents spontaneous autoimmune diabetes and restores diabetes in syngeneic islet-transplanted NOD mice. *Diabetologia* 33, 331–339 (1990).

19. Weiner, H. L., Hafler, J., Kuchroo, V. & Chen, Y. Induction and characterization of TGF- $\beta$  secreting cells. *FASEB J.* 10, A1444 (1996).

20. Becker, J. C., Czerny, C. & Brocker, B. E. Maintenance of clonal anergy by endogenously produced IL-10. *Int. Immunopharmacol.* 16, 1605–1612 (1994).

21. Gorycynski, B. M. & Vojcik, D. A. Role for non-specific (cyclosporin A) or specific (monoclonal antibodies to ICAM-1, LFA-1, and IL-2) immunomodulation in the prolongation of skin allografts after antigen-specific pretransplant immunotherapy or transfusion. *J. Immunol.* 152, 2011–2019 (1994).

22. Abramowicz, D. et al. Nonantigenic induction of transplantation tolerance in mice is associated with in vivo expression of IL-10 and mRNP. *Transplant.* 25, 318–315 (1994).

23. Blaszc, B. R., Taylor, P. A., Smith, S. & Valler, D. A. Interleukin-10 administration decreases survival in murine recipients of major histocompatibility complex disparate donor bone marrow grafts. *Blood* 83, 384–385 (1994).

24. Adlers, R. D., Staley, T. & Sidman, C. L. Differential cytokine expression in acute and chronic murine graft-versus-host disease. *Eur. J. Immunol.* 25, 333–337 (1995).

25. Lee, M.-S., Wogenen, L., Shirura, J., Oldstone, M. B. A. & Sarvenick, N. Pancreatic islet production of murine interleukin-10 does not inhibit immune-mediated tissue destruction. *J. Clin. Invest.* 93, 1332–1338 (1994).

26. Suzuki, T. et al. Viral interleukin 10 (IL-10), the human herpes virus 4 cellular IL-10 homolog, induces local anergy to alloantigen in murine skin tumors. *J. Exp. Med.* 182, 477–486 (1995).

27. Cannella, B. J., Gershwin, M. E., Sacks, C. & Fauci, A. S. IL-10 fails to abrogate experimental autoimmune encephalitis. *J. Neurosci.* 16, 735–746 (1996).

28. Nishimi, S., Tashiro, T., Murakami, M. & Honjo, T. Administration of interleukin-5 or -10 activates peripheral B-1 cells and induces autoimmune hemolytic anemia in anti-erythrocyte autoantibody-transgenic mice. *Eur. J. Immunol.* 25, 307–305 (1995).

29. Openshaw, P. et al. Heterogeneity of intracellular cytokine synthesis at the single cell level in polarized T helper 1 and T helper 2 populations. *J. Exp. Med.* 182, 1357–1367 (1995).

30. Yardi, H., de Vries, J. E., Koken, M., Van Biltenswijk, W. & Spijk, N. Serum-free medium for generation and propagation of functional human cytokines and helper T cell clones. *J. Immunol. Methods* 72, 219–227 (1984).

**Acknowledgements.** We thank E. Murphy, E. Callas and D. Polakoff for technical assistance; R. L. Coffman and L. L. Lanier for reviewing the manuscript; and J. A. Katherle for secretarial help. DNAX Research Institute of Molecular and Cellular Biology, Inc. is supported by Schering-Plough Corporation.

Correspondence and requests for materials should be addressed to M.G.R.

## Lipopolysaccharide-binding protein is required to combat a murine Gram-negative bacterial infection

Robert S. Jack, Xiaolong Fan, Martin Bernheiden, Gabriele Rüne\*, Monika Ehlers, Albert Weber†, Gerhard Kirscht†, Renate Mentelt, Birgit Fürl, Marina Freudenberg, Gerd Schmitz‡, Felix Stelter & Christine Schütt

Institut für Immunologie und Transfusionsmedizin, Ernst-Moritz-Arndt-Universität Greifswald, Salzbrückstrasse, D-17489 Greifswald, Germany

\* Institut für Anatomie, Ernst-Moritz-Arndt-Universität Greifswald, Loefflerstrasse 23c, D-17489 Greifswald, Germany

† Klinik und Poliklinik für Nuklearmedizin, Ernst-Moritz-Arndt-Universität Greifswald, Fleischmanstrasse 42–44, D-17489 Greifswald, Germany

‡ Institut für Medizinische Mikrobiologie, Ernst-Moritz-Arndt-Universität Greifswald, Lutherstrasse 6, D-17489 Greifswald, Germany

§ Max-Planck-Institut für Immunbiologie, Stübingweg 51, D-79108 Freiburg, Germany

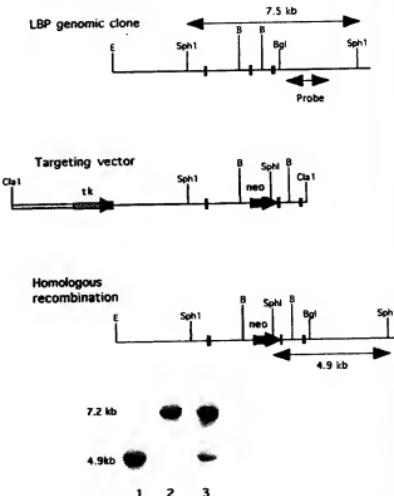
|| Universitätsklinikum, Klinische Chemie und Labormedizin, Franz-Joseph-Strauss-Allee 11, D-93042 Regensburg

An invading pathogen must be held in check by the innate immune system until a specific immune response can be mounted. In the case of Gram-negative bacteria, the principal stimulator of the innate immune system is lipopolysaccharide (LPS), a component of the bacterial outer membrane<sup>1</sup>. *In vitro*, LPS is bound by lipopolysaccharide-binding protein (LBP)<sup>2</sup> and transferred to

CD14—the LPS receptor on the macrophage surface<sup>3,4</sup>—or to high-density lipoprotein (HDL) particles<sup>5,6</sup>. Transfer to CD14 triggers an inflammatory response which is crucial for keeping an infection under control. Here we investigate how LBP functions *in vivo* by using LBP-deficient mice. Surprisingly, we find that LBP is not required *in vivo* for the clearance of LPS from the circulation, but is essential for the rapid induction of an inflammatory response by small amounts of LPS or Gram-negative bacteria and for survival of an intraperitoneal *Salmonella* infection.

The mouse LBP gene was disrupted in embryonic stem cells by homologous recombination. The mutant cells were injected into BALB/c blastocysts and a resulting mosaic male was selected to generate heterozygous and homozygous LBP-deficient animals (Fig. 1). Serum from animals heterozygous for the mutation contained LBP detectable by enzyme-linked immunosorbent assay (ELISA), but the serum from homozygous knockout mice did not (Table 1). LBP function was measured as the capacity to transfer LPS labelled with fluorescein isothiocyanate to mouse CD14 expressed on the surface of CHO cells; this was readily demonstrated in LBP<sup>+/−</sup> and LBP<sup>−/−</sup> sera but none is detectable in the serum of the LBP<sup>−/−</sup> homozygotes (Fig. 2). By these criteria, homologous recombination has resulted in a complete loss of LBP activity in our homozygous mutant animals.

Both heterozygous and homozygous LBP-deficient animals appear normal and are fertile. A preliminary analysis of tissue from all main organs of male and female LBP<sup>−/−</sup> mice at 14 weeks of age revealed no differences compared with an LBP<sup>+/−</sup> age-matched littermate. LBP may be involved in lipid transfer reactions<sup>7</sup>, so we determined the concentrations of total serum cholesterol and triglycerides in heterozygous and homozygous animals: the



**Figure 1** Maps of the mouse genomic clone containing three exons of the LBP gene (top), the targeting vector (middle) and the expected structure of the recombinant chromosome (lower). Southern analysis of three F2 littermates: homozygous LBP-deficient animal (lane 1), wild-type (lane 2) and heterozygote (lane 3).